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and
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May, 1977

Analysis of the Electron Spin Resonance of Spin Labels Using Chemometric Methods.

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Introduction

Spin labels are being used to study the structure of model membrane systems and biological membranes. (1,2) The spin labeling technique involves incorporating a nitroxide free radical (the spin label) into a membrane system and studying the free radical using electron spin resonance (ESR) spectrometry. Lipid spin labels that are diffused into a membrane orient themselves in a specific configuration and undergo anisotropic molecular motion. When this motion is rapid on the ESR time scale, the ESR spectra that are observed can be correlated with the structure of the membrane.

Molecules have been constructed so that the long axis of the molecule is parallel to one of the principal axes of the nitroxide. Anisotropic motion about the long axis of the molecule corresponds to rotation about one of the principal axes of the nitroxide. The ESR spectra of this type of molecule in a well defined inclusion crystal have been studied and synthesized in order to better understand the membrane spin labeling experiments. (3,4,5)

Studies using spin labels involve a considerable effort for the chemist in the collection and analysis of the spectra. In the past, spectra were collected as two dimensional plots on a piece of paper and the useful information extracted from the plots using a ruler and a pencil. With the introduction of laboratory computers this task has been made much easier. (6) Spectra are now collected by computer controlled spectrometers and are saved in computer compatible format (i.e., on paper tape, magnetic tape, or disks). This use of computers also allowed

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some simple data analysis techniques to be performed on the spectra. These techniques included base-line corrections and spectral smoothing. Computers have made it relatively easy to collect and store all the ESR spectra for a particular study. This paper will present examples of the use of computers to aid the chemist in the analysis of ESR spectra. The first application will involve the use of Chemometric methods to study two spin labels in different inclusion crystals. This application will demonstrate the general usefulness of chemometrics to analyzing ESR spectra. The second application will concern spin labels in a model membrane system.

Methodology

The data analysis methods used in this paper come under the general heading of Chemometrics. (7,8) The methods used are the ones that will extract features from the ESR spectra, calculate the importance of the extracted features to a particular property of the spin label, and finally, display the results. All the spectra used in this study were collected under computer control and stored in digital form as 980 data points. The 980 data points can be used as features that describe each spectrum. However, such a large number of features can present difficulties for some data analysis methods. A method that reduces the number of features describing the spectra without losing chemically useful information is clearly needed.

The method of choice in this study is the Fourier transform. Fourier transform methods have been used quite extensively in other forms of spectroscopy for a variety of purposes. (9) The effect of the Fourier transform is to condense the information of the total ESR spectrum into the low frequency end of the transformed spectrum. Figure 1 shows a typical ESR spectrum, the real and imaginary parts of the Fourier transform of the spectrum, and the power spectrum. The low frequency end of the transformed spectrum contains all the information needed to reconstruct the original spectrum via the inverse or back transform. This process is graphically presented in Figure 2. The first 64 points of the transformed spectrum are retained while the rest of the points are set to zero. The inverse transform returns the original spectrum showing that no information loss results. The spectrum resulting from the inverse transform appears to be smoother than the original spectrum because the high frequency noise has been digitally filtered by the transform. By using the Fourier transform, 64 features that completely describe the spectrum have been generated out of a spectrum of 980 data points. Once the features have been generated in this manner, the other Chemometric methods can be applied.

Two statistical methods are used to determine the importance of the generated features in modeling a property of the spin label. The property of interest in the first application is the

temperature of the spin label and the property of interest in the second application is the amount of spin label present. The first method calculates the correlation between the generated features and the property. The second method is step-wise regression analysis that determines which of the features does the best job of modeling the property with a linear model. Plots of the generated features vs. the property are also constructed as part of the analysis. All of the methods described are part of the ARTHUR pattern recognition system (10) which was used in this study.

Spin Labels in Inclusion Crystals

The first system studied using the above described methodology consisted of 3-doxyl-5α-cholestane (I) (the 4',4'-dimethyl-oxazoladine-N-oxyl derivative of 3-keto-5α-cholestane) in an inclusion crystal of thiourea. The question to be answered in this study is: can the temperature of the inclusion crystal system be correlated to the ESR spectrum?

The data set contains 16 ESR spectra of the spin label-inclusion crystal system corresponding to a range of temperatures from -82.0°C to 59.2°C. Table I lists the data analysis steps taken to analyze this series of spectra. Feature number four, generated using the Fourier transform, is found to be the most important feature in modeling the temperature of the system. Figure 3 shows a plot of the temperature vs. feature four.

Table I
Steps Taken in Data Analysis

- I Collect Spectra
- II Generate Features Using Fourier Transform
  - A Zero Fill Spectra to 1024 Points (Requirement of Fast Fourier Transform)
  - B Perform Fast Fourier Transform
  - C Select the First 64 Coefficients of the Real Part of the Transform
- III Calculate Correlation between the 64 Features and Property
- IV Perform Stepwise Regression Analysis of the 64 Features
- V Generate Plots of Features Selected in Steps III and IV vs. the Property
- VI Analyze Results

Ideally, Figure 3 should show a straight line indicating that feature four is linearly related to the temperature. The scatter of points about the line can be interpreted as meaning that the anistropic motion of the molecule is somewhat restricted. The shorter steps between feature four values at the high temperature end indicates that the rotation about the long axis of the mole-

cule is being optimized.

The second system studied consisted of the spin label lauryl nitroxide (II) (2,2,6,6-tetramethyl-4-piperidinol-1-oxyl dodecanoate) in an inclusion crystal of β-cyclodextrin. The data set for this system contains 20 ESR spectra of the spin label in the inclusion crystal corresponding to a temperature range of -196°C to 63°C. Table I again lists the data analysis steps taken in the analysis of these spectra.

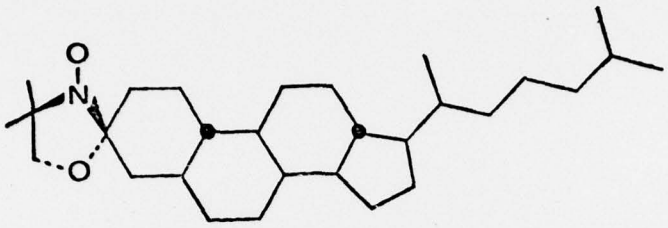
Fourier transform feature number three is shown by the stepwise regression analysis to be the most important feature in modeling the temperature of the system. Once again a linear plot of the feature and the temperature is expected. Figure 4 shows the actual plot which appears to be linear from the low temperature end (-196°C) to a temperature of about 35°C. Then the value of the feature does not get any larger. It remains nearly constant from about 35°C to 63°C. In the low temperature region, the ESR spectrum approaches the rigid glass limit. As the temperature increases, the molecule starts to rotate more freely about its long axis. At approximately 35°C the rotation about the nitroxide principal x-axis is fast enough on the ESR time scale such that the y and z contributions are averaged out. A further increase in temperature has no additional effect on the anistropic motion.

It is interesting to compare both spin labels in their rigid matrices. The lauryl nitroxide is able to rotate quite freely and reaches an optimum value. The 3-doxyl-5α-cholestane is not able to rotate as freely as the lauryl nitroxide and appears not to reach an optimum value. This difference in rotation can be accounted for by the structure of the molecules. Lauryl nitroxide is a long, cylindrical-shaped molecule, while the 3-doxyl-5α-cholestane is a rectangular shaped molecule. It is easier for the cylindrical molecule to rotate about its long axis in a cavity in a matrix than it is for the rectangular-shaped molecule.

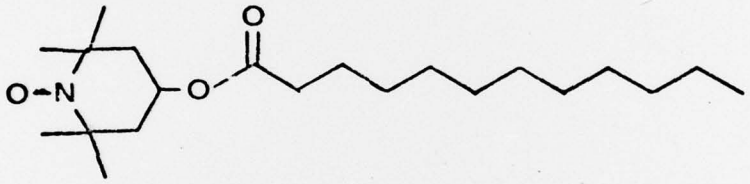
Now that the Chemometric methods have been shown to be useful in the study of spin labels in well-defined inclusion crystals the methods can be used in the study of spin labels in a model membrane system. The last part of this paper will deal with the application of Chemometric methods to the study of such a model membrane system.

Spin Labels in Model Membrane Systems

The model membrane system studied is the cytochrome oxidase protein containing spin labeled phospholipids. The spin label used is 16-doxyl steric acid (III) (the 4',4'-dimethyloxazoladine-N-oxyl derivative of 16-keto stearic acid). Figure 5 shows the spectra of representative samples of the cytochrome oxidase protein with different concentrations of phospholipids. The amount of lipid in each sample is expressed as the ratio of mg of phospholipid per mg of protein. The sample in Figure 5a has a ratio



Structure I



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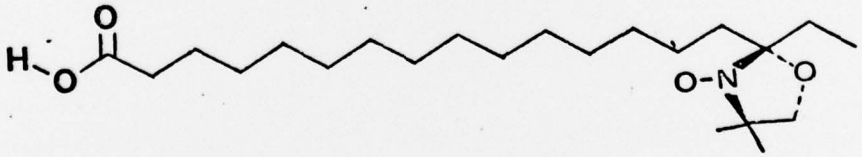

Structure III

of 0.10; Figure 5b corresponds to a ratio of 0.24; and Figure 5c is 0.73. The ESR spectrum of the sample with the lowest lipid content (Figure 5a) is characteristic of strong immobilization of the spin labels while the spectrum of the sample with the highest lipid content (Figure 5c) is characteristic of a more mobil spin label. (11) The question to be answered in this experiment is: is it possible to quantify the amount of each kind of spin label in a composite system as shown in Figure 5b?

The data set includes eight spectra of samples of varying amounts of the protein and spin labeled phospholipid. The feature generation methodology used is the same as described in the previous examples. The property of interest in this example is the amount of immobilized lipid present in the model membrane system. By using stepwise regression analysis it is possible to arrive at an equation to calculate the amount of the lipid present.

By using this equation, it is possible to look at the Fourier transform of an ESR spectrum of the membrane system and calculate the amount of immobilized lipid present. A practical proof of the validity of this equation is to synthesize an ESR spectrum for the series studied using spectra of the immobilized spin label and the mobil spin label. Since the equation was developed using the Fourier transform to the ESR spectra, they will be used in place of the spectra. The Fourier transform of the immobilized spin label spectrum (Figure 5a) is multiplied by the calculated scale factor and the result is added to the Fourier transform of the mobil spin label scaled by the calculated factor. Then the inverse transform is applied to this composite to give the spectrum. In this case the Figure 5b is the spectrum that is being synthesized. The scale factor for the immobilized spin label is 0.24, and the factor for the mobil spin label is 0.76. This process is shown graphically in Figure 6. The resultant synthetic spectrum appears smoother than the experimental spectrum because the high frequency noise has been digitally filtered.

In this application Chemometric methods were used to show that certain ESR spectra of a model membrane system are a composite of spectra of an immobilized spin label and a mobil spin label.

Conclusions

Chemometric methods have been used to analyze experimental ESR spectra. The methods have provided additional insight into the processes involved in putting a spin label into an inclusion crystal. They have also been used to examine the ESR spectra resulting from spin labels dispersed in a model membrane system. Chemometrics does provide a powerful tool to aid the chemist in the analysis of ESR spectra of spin labels in model membrane systems.

Acknowledgments

We wish to acknowledge Drs. Patricia Jost and O. Hayes Griffith for kindly providing us with the ESR spectra used in this study. We also wish to acknowledge the financial support of the Office of Naval Research under Contract No. N00014-75-C-0536.

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Legends for Figures

Figure 1. Typical ESR spectrum and its Fourier transform.

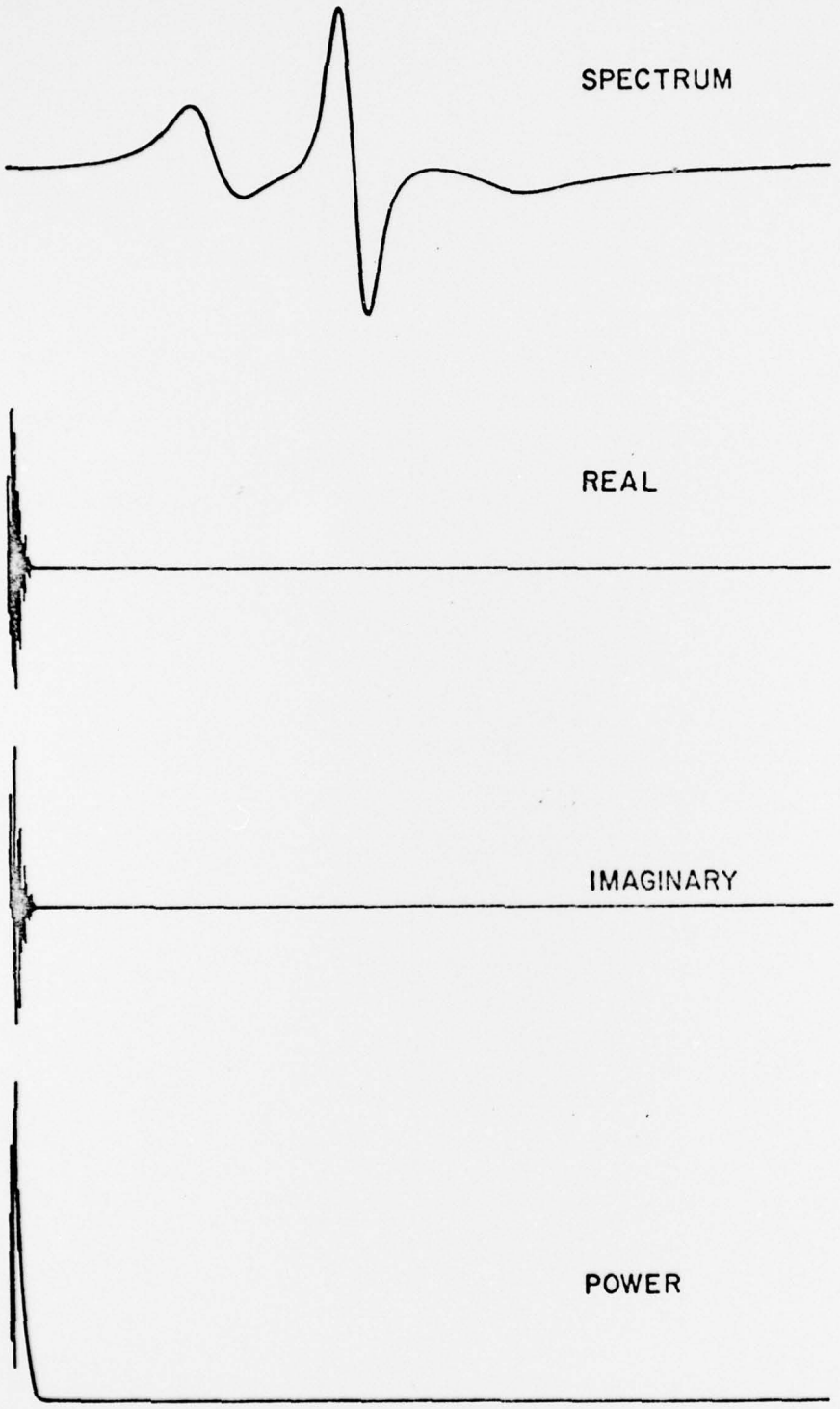
Figure 2. Graphical demonstration that only the first 64 points of the Fourier transform of an ESR spectrum are needed to regenerate the spectrum from the transform.

Figure 3. Plot of the Fourier transform generated feature number four (the ordinate) vs the temperature of the system (the abscissa) in sample one.

Figure 4. Plot of the Fourier transform generated feature number three (the ordinate) vs the temperature of the system (the abscissa) in sample two.

Figure 5. ESR spectra of spin labeled phospholipids in cytochrome oxidase (see text).

Figure 6. Graphical presentation of the generation of a composite ESR spectrum by using scaled amounts of Fourier transform of two ESR spectra.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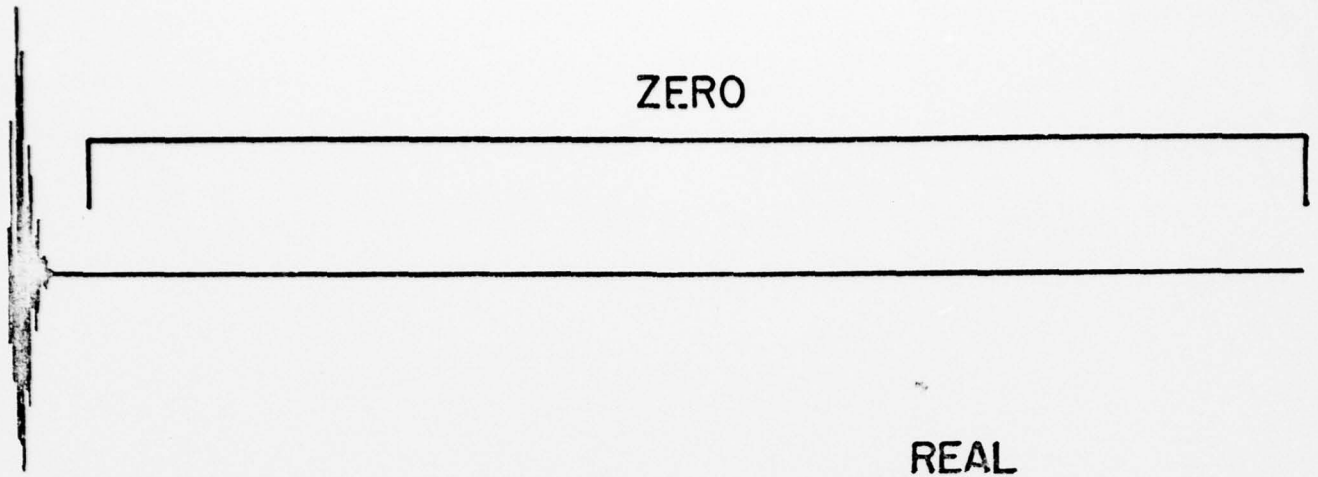

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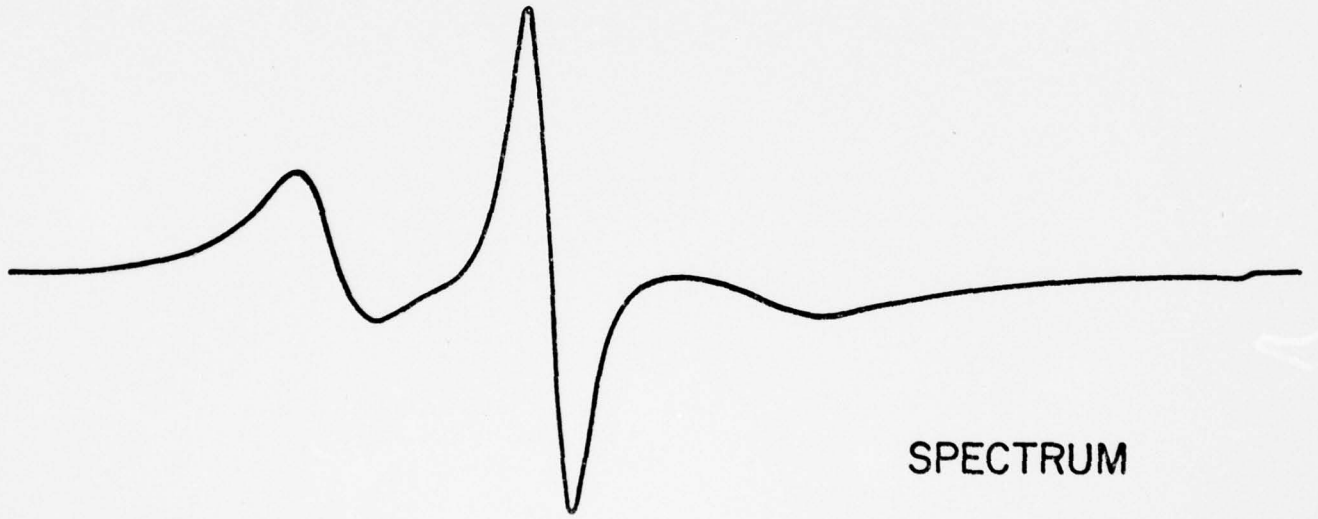

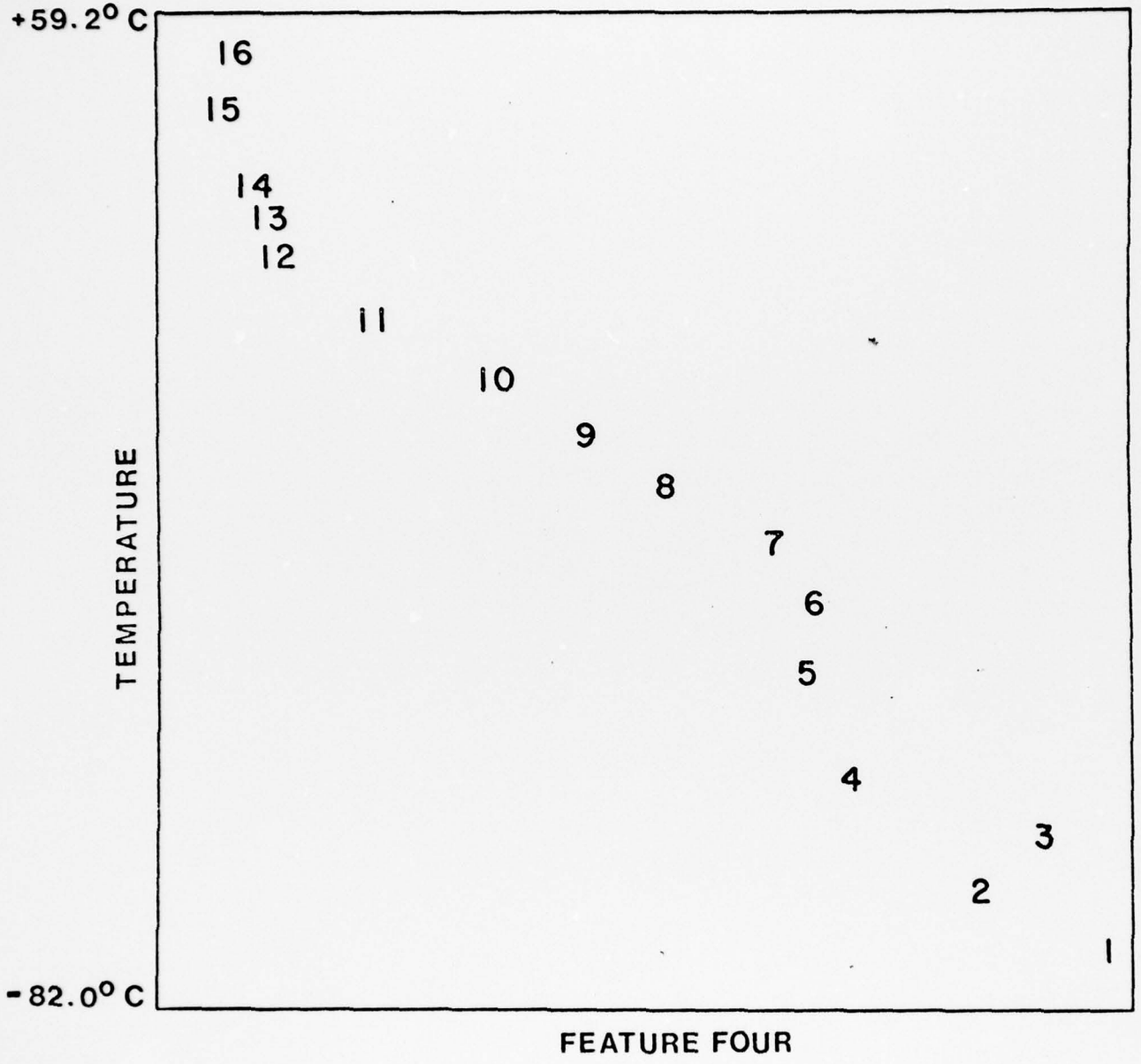
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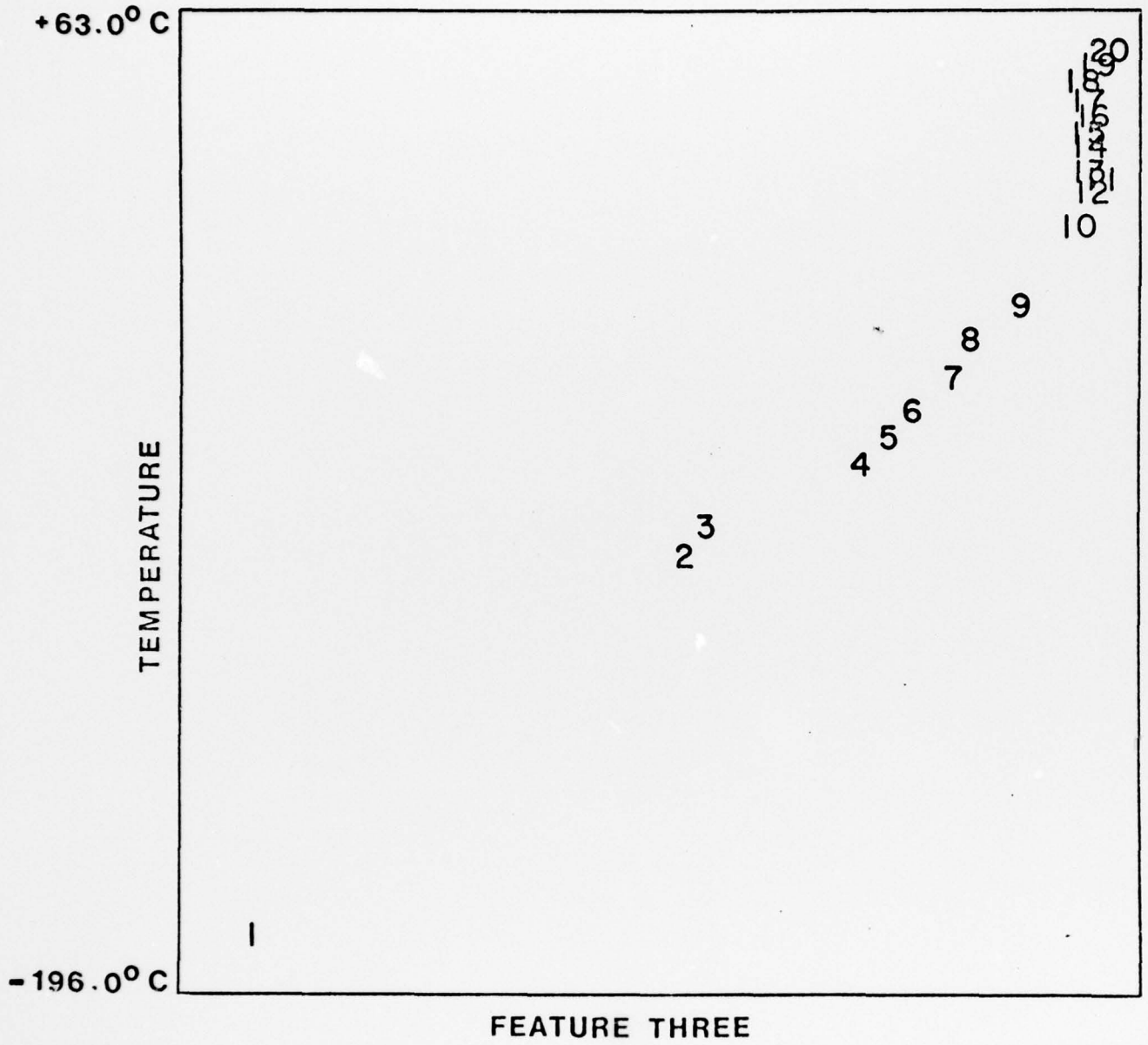
TEMPERATURE
FEATURE FOUR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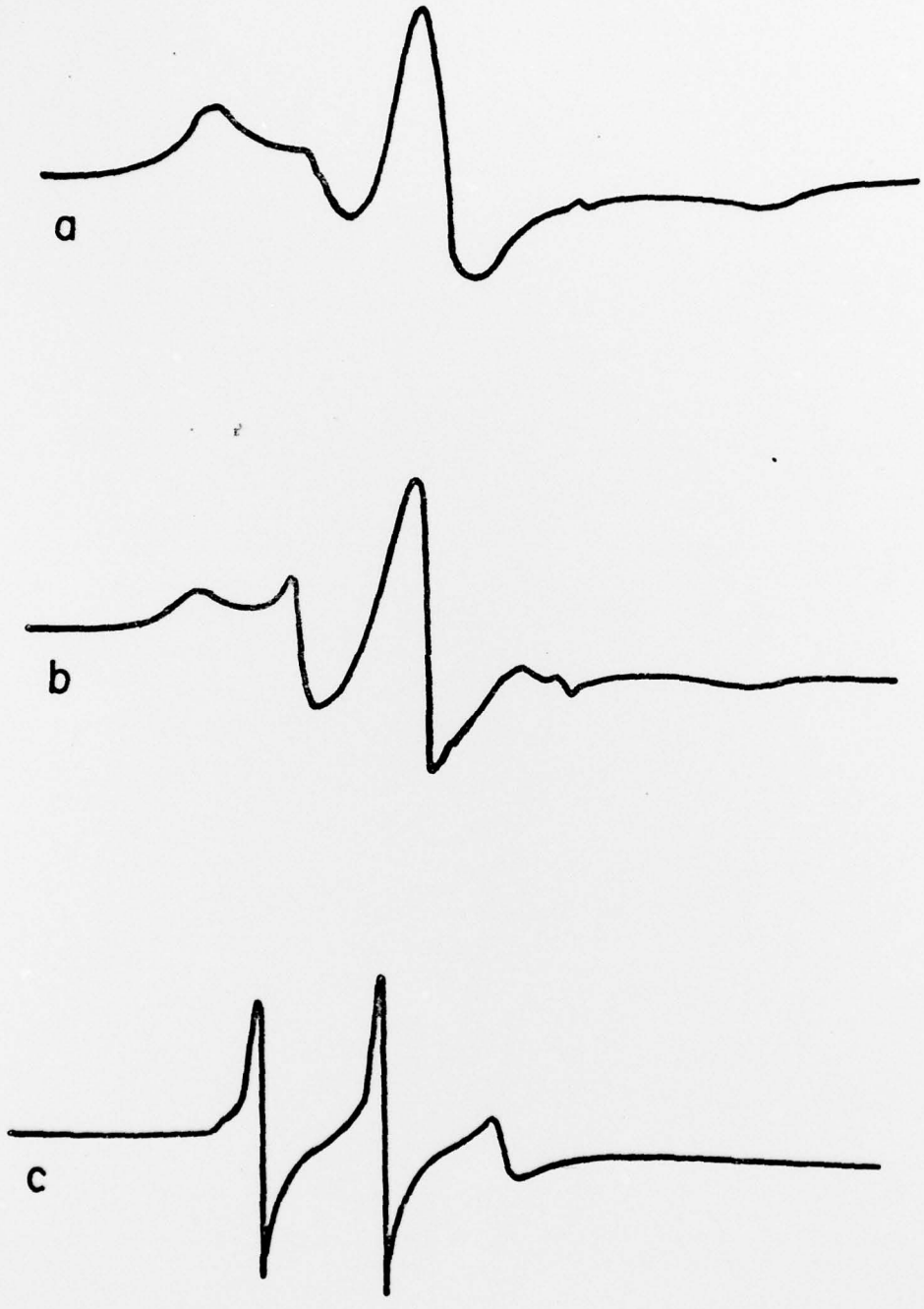
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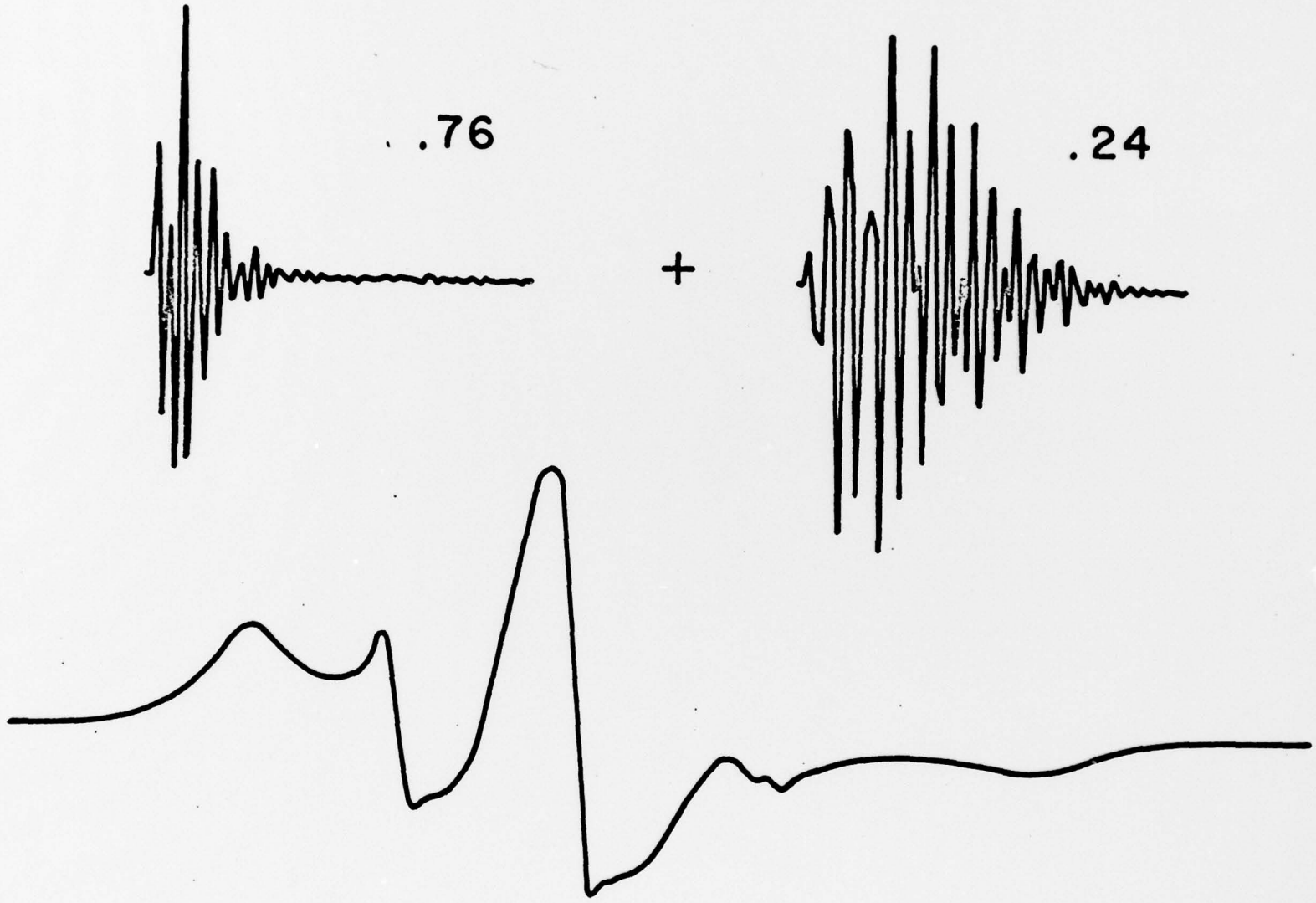
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